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DEVELOPMENT OF A RHYZOBIAL INOCULANT FOR SAINFOIN

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Development of a Rhizobial Inoculant for Sainfoin

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a) Summary

Thirty-six strains of Rhizobium were screened in a series of experiments for their ability to nodulate sainfoin. The objective was to identify strains with a superior potential to nodulate and ultimately fix nitrogen in sainfoin (*Onobrychis viciaefolia*). The 36 original strains were acquired from collections at Philom Bios, Saskatoon, SK, Agriculture and AgriFood Canada, Quebec, University of Minnesota, and field collections. Of the 36 strains, two were selected for the improved N nutrition in sainfoin inoculated with the strains. Both strains were from the AAFC collection and were originally isolated from arctic soils. This cold climate selection may provide an advantage for inoculants in western Canada. This study represents the first phase in selecting a superior rhizobial strain. The next step will be to evaluate directly the N-fixation capability of sainfoin inoculated with the strains, evaluate how competitive the strains are in different field soils, and quantify nodule occupancy by these introduced strains. The perennial nature of sainfoin makes it necessary to evaluate the effectiveness of N-fixation over multiple growth seasons if sainfoin can become an economical alternative to alfalfa

b) Introduction

Crop diversification is a goal for most Saskatchewan producers. Growing a variety of crops minimizes the risk of widespread crop failure under extreme environmental conditions. Most often, crop diversification is associated with production of annual crops, but as the numbers of head of cattle increase in the province, the need to grow different types of forages will become more of a priority. Initiatives that will contribute to the expansion of the forage industry include the goal of increasing beef cow herds to a projected 3.3 million head by the year 2010, up from 2.2 million head in 2000 (SAC, 2000). Clearly, land usage will need to be optimized in order to feed these higher herd numbers. In addition, the Saskatchewan government has implemented permanent cover initiatives to convert marginal farmlands back to more permanent pasture conditions. The permanent cover incentive is aimed at resource conservation, as well as soil sequestration of carbon as one strategy to reduce agricultures' contribution to greenhouse gas emissions.

In addition to developing new crops and crops varieties, there is a need to optimize agronomic practices associated with the crop in question. Sainfoin (*Onobrychis viciaefolia* Scop.) represents a potential forage legume alternative to the primarily alfalfa and sweet and red clovers grown in Saskatchewan. It is currently grown in Saskatchewan but in very small acreages. Like alfalfa it can biologically fix nitrogen from the atmosphere and is of excellent quality and palatability. In hay production its yield is often as high as alfalfa when one cutting is compared, but about 80-90% of alfalfa when two cuttings are compared (Alberta Forage Manual, 1996). It also tends to be short-lived in the field but has the potential for being long-lived if managed properly (Alberta Forage Manual, 1996). Despite its somewhat lower productivity, it does have some desirable characteristics that make it an attractive alternative

crop to alfalfa. Foremost, it does not induce bloat in grazing animals like alfalfa does, so can be grazed in pure stands (Hanna et al., 1979). Studies in Alberta have shown excellent results when cattle are grazed on a sainfoin/alfalfa mixed pasture (Berg, 2003). Furthermore, sainfoin is immune to the alfalfa weevil and could be used in areas where the weevil is a problem. Like alfalfa, it can be used for honey production. It is especially well adapted to the dry calcareous soils that predominate in Saskatchewan, and is somewhat more drought and cold tolerant than alfalfa. Unlike alfalfa, the stems remain succulent as the plant matures, so the quality of the forage does not decrease as rapidly.

One of the biggest shortcomings for widespread sainfoin production is the limited availability and poor quality of the rhizobial inoculants. To quote the Alberta Forage Manual (1996) "nitrogen-fixing bacteria have been short-lived or ineffective so that nitrogen fertilization may be required". Many strains are very effective initially but are not competitive with other soil bacteria (Peter Graham, Univ. of Minnesota, personal communication). A search of inoculant companies revealed only one (Nitragin, Milwaukee, WI) that produced inoculant for sainfoin. The company was recently taken over by Liphatech Worldwide, and it appears may not market the inoculant any longer. Clearly, finding a source for inoculant is challenging.

Improper or ineffective inoculation and hence poor nitrogen nutrition could account for the short-lived nature of the crop as well as its sometimes weak competitive ability with weeds (Hume et al., 1985). Sainfoin was introduced to North America from Europe and Asia where it has been cultivated for hundreds of years. Because it is an introduced crop, it is very unlikely that native N-fixing bacteria are present in our soils. Furthermore, because none of the local western Canadian inoculant producers manufacture a sainfoin inoculant, those that

can be acquired may not be adapted to our soil and climatic conditions, accounting in part for their "short-lived" or "in-effective" nature.

The objective of this study was to screen rhizobia from existing cultures and from field collected sainfoin and vetches for their ability to nodulate sainfoin. The species of *Rhizobium* responsible for nodulating sainfoin has not been identified specifically but is thought to be a consortium of rhizobia that cross nodulate bean, vetches and some other species not found in Saskatchewan. This proposal represents a first step toward selecting a superior strain of *Rhizobium* that could lead to better and sustained nitrogen fixation in sainfoin.

c) Methods

Sample collection and purification:

A variety of strains of rhizobia were obtained from the rhizobia collections at: Philom Bios Saskatoon, SK (M. Leggett); the University of Minnesota (P. Graham); and the Agriculture and Agrifood Canada laboratory in St. Foy, Quebec (D. Prevost). Strains that nodulate bean were targeted from the collections. Upon receipt, the cultures were streaked onto yeast mannitol agar (YMA) plates containing a congo red (CR) indicator (25μg mL^{-1}) and incubated in the dark. Rhizobia generally do not absorb CR, but produce white, opaque colonies. Most other bacteria absorb the red indicator. This initial culturing was done to confirm purity of the cultures and confirm that the bacteria were rhizobia.

Native vetch plants with their associated root systems were collected from various locations around Saskatchewan. Root systems of individual vetches, with the associated shoots, were dug up by hand and placed in plastic bags. Plants that were collected remotely were shipped via bus overnight. Plants were stored refrigerated for several days until the

rhizobia could be isolated from the nodules. A number of collection plants did not have nodules on the root systems, indicating they were not actively fixing nitrogen. These specimens were excluded from the study as it was not possible to isolate nodular rhizobia.

After removal from the root system, several nodules per plant were surface disinfected by immersing for 10 s in 95% (v/v) ethanol, followed by 2 min in sodium hypochlorite (3 % v/v). Nodules were rinsed in five changes of sterile, distilled water.

Surface disinfected nodules were crushed in a drop of sterile water in a petri dish. One loopful of the nodule suspension was streaked onto a yeast mannitol agar (YMA) plate containing CR indicator (25μg mL^{-1}). Plates were incubated at room temperature in the dark. Those colonies that did not absorb CR were selected as presumptive rhizobia. Single colonies from the selective CR agar were streaked onto YMA plates and these secondary plates used to make stock solutions for further tests.

A total of 36 isolates were included in the initial screening (Table 1): 11 isolates from Philom Bios; 12 from U. of Minnesota; 6 from AAFC; and 7 from native vetches. The isolates were labeled 1 through 36 and this numerical designation used for further identification of the isolates throughout the experiments.

Sample preservation:

Stocks of each of the cultures were prepared for long-term storage. 50 mL of glycerol solution (10% glycerol plus 0.01% Tween 80) was prepared and autoclaved for 30 min. A loopful of culture was aseptically added to the glycerol. The suspension was vortexed to attain a homogeneous suspension. Aliquots of the suspension were transferred to Nalgene cryogenic

vials and frozen at –80° C. Prior to use, a stock vial was retrieved from the freezer and allowed to thaw 20 min at room temperature.

Growth pouches:

Growth pouches are compact systems that enable short-term growth of large numbers of plants for screening purposes. They can be autoclaved. They are composed of a flat, square plastic bag with a paper liner in the interior. The top of the bag is open to the atmosphere. The top of the liner is folded to form a trough where seeds or seedlings can be placed. Nutrient solution is added to the bag and is wicked up the paper liner, supplying nutrients to the seedling. The bag is clear to allow roots (and nodules) to be observed.

Single colonies from the YMA cultures were selected and grown in liquid culture (yeast mannitol broth, YMB) for 5 days to 2 weeks, depending on the growth rate of the culture. Cultures were grown until the culture flasks attained a milky, yellow-white, opaque appearance. Ten-fold serial dilutions of the cultures were prepared in sterile, deionized, distilled water over the range of 10^{-1} to 10^{-12}.

Growth pouches were prepared for each strain. Sterile pouches were filled with 200 mL of sterile N-free medium (Fahraes, 1957). For each strain 10 dilutions (10^{-1} through 10^{-10}) and a *Rhizobium*-free control after each set of four replicates of a dilution were tested (50 pouches per strain).

Sainfoin seed was manually removed from the pod and scarified by rubbing the seed with sand paper. After scarification the seed was surface disinfected with a combination of ethanol (95%) for 10 s, and bleach (3%) for 2 min. Seeds were pregerminated in the dark on filter paper moistened with sterile water. After the radical emerged from the seed (1to 3 days) two

seedlings were transferred into the seed trough of a sterile growth pouch containing sterile N-free nutrient media. One mL of the appropriate dilution of the test *Rhizobium* strain was pipetted onto the pregerminated seed in the growth pouch. The uninoculated controls received 1 mL of sterile water. Growth pouches were moved to a growth chamber (16 h photoperiod, 20 °C/15°C day/night temperature) and maintained for 4 wk before scoring for nodulation. Nutrient solution was replenished after 2 wk. Pouches were watered with sterile water as needed.

Sainfoin roots were scored for the presence or absence of nodules after 4 wk of growth. This data was subjected to a statistical analysis (Most Probable Number count; MPN) that identifies the most probable number of colony forming units that were able to nodulate the plant (Somasegaran and Hoben, 1994). The statistical procedure uses the nodulation score (presence or absence of nodules), the number of replications, the number of dilutions steps, the lowest dilution where nodules are found and the volume of inoculant applied to the plant to calculate an estimate of the number of viable and infective rhizobia nodulating a plant.

Enumeration of stock cultures:

For each experiment (growth pouches, Leonard jars and soil-based) triplicate YMA plates for dilutions 10^{-5} to 10^{-12} were spread. Plates were incubated at 20°C and colonies counted after they had sufficient time to grow. The culture time varied from 3 days to 2 weeks because of the differences in growth rates. The numbers of colony forming units per mL of culture added in each experiment was back calculated from the growth on the dilution plates.

Leonard jars:

Nine rhizobial isolates were selected from the original 36 for further testing. Eight of the strains performed well in the growth pouch experiments, in that high MPN scores were identified. This indicates that high numbers of bacteria of a particular strain were effective in nodulating the sainfoin. A strain that did not nodulate sainfoin in the growth pouches was included as a negative control, plus an uninoculated check.

Leonard jars are a semi-sterile, soil-less system used to grow plants (Somasegran and Hoben, 1994). They are larger than growth pouches and hence can be used to grow plants for a longer period of time. Furthermore, roots grow in sand (or vermiculite) and hence are more comparable to soil-based systems. The Leonard jar assembly involves inverting a bottle (usually a beer bottle) into the mouth of a mason jar. The bottom of the bottle is cut off. The mason jar acts as a reservoir for N-free nutrient solution. 700 mL of N-free nutrient solution (Fahraeus, 1957) was added to each reservoir. A length of cotton lamp wick was positioned to run from the floor of the mason jar up through the neck of the bottle to within a cm of the top surface of the inverted bottle. The wick was held in place in the neck of the beer bottle with a piece of cotton batting. With the wick in place, the inverted bottle was filled with clean sand to approximately 1 cm of the top surface. The sand is the rooting media for the plant, the reservoir holds the nutrient solution and the wick acts to draw solution from the mason jar into the sand. After the Leonard jars were assembled, the entire apparatus (excluding the top surface – leaving the sand exposed) was wrapped in brown paper to block light. The top of the assembly (exposed sand) was covered with aluminum foil. This allows the sand surface to be exposed without unwrapping the entire apparatus. The jars were autoclaved for 45 min, and allowed to cool to room temperature.

Two surface disinfected, scarified seeds were seeded approximately 0.5 cm deep into the sand. One mL of the rhizobial culture was pipetted onto each seed. The isolates used to inoculate the seed were grown in YMB as described previously. Numbers of bacteria in the original cultures were enumerated using dilution plate counts as described above. After the sainfoin was seeded and inoculated, the foil cap was replaced over the Leonard jar and remained in place until the seedlings had emerged. After emergence the foil cap was removed. Water lost through evapotranspiration was replaced as needed by adding sterile water to the reservoir. Sterile, nutirent solution was replaced weekly. The Leonard jars begin as sterile units, but because they are open to the atmosphere do not remain sterile. They provide an environment with very little microbial competition for assessing microbial inoculants, a controlled source and amount of nutrients, and unlike pure hydroponics systems, a rooting media comparable to at least some soils.

Two experiments were established with the Leonard jars, one at a thermo-photoperiod of 12°C, 16 h day; 8°C, 8 h night and the other at 20°C, 16 h day ; 16°C, 8 h night. Plants in the cool temperature chamber did not grow and the experiment was terminated after 6 weeks but no data was collected. None of the roots systems were nodulated although the plants remained alive. Considering that the objective of the experiment was selection of a superior rhizobial strain, the fact that no nodules developed with any of the strains indicated that this objective was not achievable under the cool growth conditions.

Plants grown in the warm temperature regime, were also grown for 6 weeks and then harvested. Roots were scored for nodulation, nodules harvested, counted and weighed. Root and shoot materials were collected separately and dried in a forced air oven (60 °C). After grinding, the tissues were acid digested (Thomas et al., 1967) and analysed for N and P content

on an autoanalyser. Nutrient uptake was calculated by multiplying the nutrient content in the tissue by the total mass of the particular tissue.

Soil-based experiments:

The four best performing strains from the Leonard jar experiment were further tested in a soil-based growth chamber experiment. The soil-based experiment enabled assessment of nodulation and potential nitrogen fixation under conditions where microbial competition can occur. Field soil was collected from a field in the Brown soil zone near Elbow, SK. The soil has a clay loam texture, and electrical conductivity (1:2 soil water extract) of 0.3 mS cm^{-1}, organic matter content of 2%, 6.7 kg N ha^{-1}, 55 kg ha^{-1} P_2O_5, 1005 kg ha^{-1} K and 30 kg ha^{-1} of S. The soil was air-dried and sifted (4 mm screen) to remove large pieces of organic matter and soil lumps. Soil was placed into 20 cm (dia) pots. Pregerminated sainfoin seedlings were transplanted into each pot and 1 mL of the appropriate bacterial culture pipetted onto the root system of each seedling. The growth chamber was set at 22°C/18°C day/night temperature. Photoperiod in both experiments was 14h. Plants were grown for 10 weeks. Harvested plants were dried in a forced air oven (60°C) and separated into roots and shoots. Shoots and roots were analysed for N content by acid digesting the tissue and analysing the digests on an autoanalyzer.

Statistical analyses:

Both the Leonard jar and soil-based experiments were established in completely randomized designs with six replicates. Analysis of variance was performed and means separated using least significant differences (lsd) at an α=0.05.

d) Results and Discussion

Sainfoin growth:

Sainfoin is a difficult plant to work with. We tried several screening methods for the initial screening of the 36 original strains. In our first attempts with the growth pouches the very fine root systems dried out very rapidly in the growth pouches. We tried culturing the seedlings in NifTal-tubes (Somasegran and Hoben, 1994) where the seedlings are grown enclosed in sterile tubes on agar slants. Our tubes routinely became overrun by fungi, the fungi presumably originating from within the seed. We continued with the growth pouches eventually developing a reliable, repeatable protocol with the pouches. The pouches required daily watering, and needed to be rotated daily so that one strain would not dehydrate because of its position in the incubator. We also placed open containers of water in the chamber to increase humidity levels and turned off the circulating fans.

In addition to the challenges with growing the plants, the seed had very poor germination and emergence especially when seeded into sand or soil. Germination tests performed in the lab in petri dishes lined with filter paper, revealed approximately 80 % germination. However, we typically achieved about 10% emergence of the seedlings in the Leonard jar and soil-based experiments. Transplanting pregerminated seeds into the Leonard jars and pots was not successful. We had only about 10% survival of the transplants. Our solution was to overseed the Leonard jars and pots – seeding 10 seeds for every one seedling and removing any extra seedllings that emerged.

These challenges with growing the plants will translate into similar difficulties for farmers growing sainfoin, in that not unlike other small-seeded forage legumes, it can be difficult to establish. Compounding this is the nature of the seed itself. Each seed is contained

within an individual pod. While the pod itself is not impenetrable, the true seed coat must be mechanically scarified to allow the seed to imbibe water. The seed pod constitutes another physical barrier to water entering the seed. The seed pod also constitutes a barrier between the seed and any applied inoculant. We manually removed the seed pod, and mechanically scarified the seed coat with sand paper. The poor emergence may have been due to incomplete scarification of the seed coat. Although the seed germinated relatively well on filter paper, the soil-seed contact may not have been good enough to allow complete imbibition of the seed. For farmers, a reliable method for scarifying large quantities of seed will be necessary. As with other small-seeded forages, it appears that seed-bed preparation also will be instrumental in proper establishment.

Growth pouches:

All of the rhizobial strains tested grew well in culture (Table 1). Industry standards for commercial *Rhizobium* products are a minimum guarantee of 7.4×10^8 to 1×10^9 cfu g^{-1} peat inoculant (Philom Bios Inc.; Becker Underwood) and 7.5×10^8 cfu mL^{-1} liquid inoculant (Becker Underwood). All of the cultures we applied exceeded the industry standard. Furthermore, industry standards are for a unit of inoculant (g or mL) being applied to multiple seeds. For example, a 490 g bag of TagTeam (a mix of *Rhizobium* and Jumpstart) for alfalfa, will inoculate 220 pounds of seed (Philom Bios Inc., TagTeam product label, 2007). In our study, we are applying 1 mL of inoculant to a single seed to increase the probability that nodulation will occur if the potential exists in a particular strain. Only a single viable, infective cell is necessary to nodulate a plant. Even though some culture numbers were higher

for some strains than other strains, ensuring that at least 1 x 10^9 cells are available for infection ensures that this level of probability for infection is very high.

Of the 36 strains screened, 20 of them did not nodulate sainfoin at all. In the MPN protocol the appearance of even a single nodule is recognized as a positive infection. Zeros for the MPN score indicate that no nodules were observed. Nine of the strains (5, 6, 10, 12, 15, 23, 24, 26 and 27) were selected for further study based on the relatively high MPN numbers. An additional strain (4) was included as a negative control We also specifically selected strains from each of the three culture collections (University of Minnesota, Philom Bios and Agriculture and Agrifood Canada) to represent the potentially diverse environments the strains originate from. The AAFC strains, in particular, are selected from arctic soils and are adapted to cold conditions. As a group these strains performed the best overall in the initial screening and four of the six AAFC cultures were selected for further testing. None of the native collections nodulated sainfoin. It appears that at least in the regions we collected from, native rhizobia for sainfoin infection do not exist. Because sainfoin is an introduced crop we would not expect specific native populations to have evolved, but did expect that some degree of cross inoculation with the rhizoia that infect vetches.

Table 1. Most probably number (MPN) and plate counts of *Rhizobium sp.* screened for their ability to nodulate sainfoin. Plate counts are numbers of colony forming units (cfu) per mL of inoculant.

Strain	Origin[1]	MPN	Plate Count (cfu mL^{-1})	Strain	Origin[1]	MPN	Plate Count (cfu mL^{-1})
1	UM	625	8.83×10^{14}	**21**	PB	6	1.89×10^{10}
2	UM	27	4.63×10^{11}	**22**	AAFC	425	9.20×10^{14}
3	UM	0	5.43×10^{12}	**23 x**	AAFC	125	3.34×10^{14}
4 *	UM	0	9.33×10^{8}	**24***	AAFC	925	1.76×10^{15}
5*	UM	1100	1.86×10^{15}	**25**	AAFC	425	1.91×10^{15}
6*	UM	925	2.33×10^{15}	**26***	AAFC	550	3.67×10^{15}
7	UM	0	4.53×10^{14}	**27***	AAFC	21000	4.75×10^{11}
8	UM	0	7.10×10^{13}	**28**	V	0	4.34×10^{13}
9	UM	0	2.90×10^{15}	**29**	V	0	6.29×10^{11}
10*	UM	21000	$>5 \times 10^{15}$	**30**	V	0	1.46×10^{10}
11	UM	0	1.57×10^{10}	**31**	V	0	8.20×10^{14}
12*	UM	925	1.46×10^{10}	**32**	V	0	6.25×10^{12}
13	PB	0	7.80×10^{9}	**33**	V	0	9.13×10^{10}
14	PB	10	1.04×10^{10}	**34**	V	0	3.37×10^{14}
15*	PB	550	2.71×10^{15}	**35**	V	0	8.10×10^{11}
16	PB	0	4.64×10^{12}	**36**	V	0	5.55×10^{12}
17	PB	0	1.91×10^{11}				
18	PB	6	2.10×10^{10}				
19	PB	0	6.03×10^{9}				
20	PB	0	3.37×10^{10}				

[1]UM = University of Minnesota, PB = Philom Bios Culture collection, AAFC = Agriculture and AgriFood Canada, V=field collected vetch

* Strains selected for further study. Strain 4 is included as a negative control.

Leonard jars:

Numbers of rhizobia of the 10 strains used to inoculate sainfoin in the Leonard jar experiment were all in the same magnitude (Table 2). Although these concentrations are lower than those applied in the growth pouch screening they still exceed the industry standard when calculated on a per seed basis. Between 10^8 and 10^9 cfu per seed were applied to each seed compared to 1 g of inoculant inoculating approximately 0.45 pound of alfalfa seed (Philom Bios Inc., TagTeam product label, 2007). It is impossible to apply exactly the same numbers of cells because of differences in growth rates of the strains. We have to rely on the appearance of the cultures as a guide. The AAFC strains also grew at a much slower rate than the other strains, so these strains were cultured about 10 days before the other strains so that all of the strains were in adequate numbers the day the experiment was set up.

In general those plants inoculated with the AAFC strains had the best root growth (Table 3). All of these strains except #26 produced more root biomass than the uninoculated control. Shoot growth did not mirror root growth. Instead, those inoculated plants that produced the most root biomass tended to produce the least shoot biomass, and vice versa. Plants inoculated with strain #23 are the most obvious example of this. Plants inoculated with strain #15 were the main exception, as well as the uninoculated controls. Plants inoculated with strain # 15 were among the highest in both root and shoot biomass production.

Table 2: Origin of culture and numbers of colony forming units in the broth cultures used to inoculate sainfoin in the Leonard jar and Soil-based experiments. Only four of the strains tested in the Leonardjar experiment were tested in the Soil-based experiment.

Strain	Origin	Leonard Jar Concentration (cfu mL^{-1})	Soil-based Concentration (cfu mL^{-1})
4	UM	$3x10^8$	-
5	UM	$4x10^8$	-
6	UM	$2x10^8$	-
10	UM	$5x10^8$	-
12	UM	$>1x10^9$	$4x10^8$
15	PB	$>1x10^9$	-
23	AAFC	$>1x10^9$	$>1x10^9$
24	AAFC	$3x10^8$	$3x10^8$
26	AAFC	$7x10^8$	$>1x10^9$
27	AAFC	$2x10^8$	-

Table 3: Productivity of sainfoin grown in Leonard jars with selected rhizobial strains. Plants were grown for 6 weeks. Numbers are averages per plant.

Strain	Root Weight (mg)	Shoot Weight (mg)	Nodule Weight (μg)	Nodule Number
4	31.1ab[+]	23.3bc	1.1c	0.4c
5	26.1b	31.3b	12.5b	30.3a
6	20.7b	33.2b	13.4b	14.0b
10	26.9b	40.0a	48.5a	46.0a
12*	25.6b	32.1b	9.6b	8.2b
15	30.7ab	45.1a	11.7b	7.0b
23*	43.3a	29.5b	22.4b	65.4a
24*	40.7a	14.5c	25.7b	40.0a
26*	34.1ab	30.9b	18.0b	11.1b
27	38.5a	30.8b	14.5b	8.3b
uninoculated	17.9b	29.1b	0c	0c

* Strains selected for further study.

[+]Within a column, numbers followed by the same letter are not significantly different according to least significant difference test ($P \geq 0.05$).

Except for strain # 4, which was included as a negative control, all of the plants grown in the Leonard jars developed nodules (Table 3). Only a single plant incoulated with strain #4 was nodulated. Nodule numbers and nodule weights were very variable, to the extent that differences among the strains were not detectable. The lack of nodules detected on plants inoculated with the negative control strain and the uninoculated control plants, confirms that we did not experience cross-contamination among strains in the Leonard jar study.

Interestingly, the more massive root growth achieved by the plants inoculated with the AAFC strains did not translate into higher N contents in the roots. Except for the plants grown with strain #23, all of the plants effectively transported N from the roots to the shoots. Plants inoculated with strain #23 were superior to the others in terms of root production and N content in the root and shoot. Together these factors resulted in the total N acquisition by these plants being higher than all of the others (Table 4). It is possible that, particularly in the slow growing AAFC strains, not enough time had passed to enable the plants to fix N at their peak capacity.

There is some evidence that some rhizobial strains can improve P uptake by plants (Chabot et al., 1996; Antoun et al., 1998). Chabot et al. (1996) inoculated lettuce and maize with two strains of *R. leguminosarum* which were selected for their P solubilization and found these two strains promoted root colonization, growth and increased significantly in P concentration. In our study P contents in roots and shoots and total P uptake by plants inoculated with the different strains did not vary, except that all were higher than the uninoculated controls. This is probably indicative of the overall better health of the effectively nodulated plants, rather than a specific ability of the strains to improve P uptake.

Table 4: Nutrient (Nitrogen and Phosphorus) contents of sainfoin grown in Leonard jars and inoculated with selected rhizobial strains. Plants were grown for 6 weeks. Numbers are averages per plant.

	N content (mg g^{-1} tissue)		N uptake (mg $plant^{-1}$)	P content (mg g^{-1} tissue)		P uptake (mg $plant^{-1}$)
Strain	**Root**	**Shoot**		**Root**	**Shoot**	
4	29.1ab[+]	33.5b	1686b	26.7a	19.4a	1282a
5	31.3ab	45.7a	2247ab	23.2a	9.7ab	909a
6	34.1ab	50.4a	2379ab	18.3a	13.6ab	830a
10	19.6b	34.9b	1923b	19.3a	9.2ab	887a
12*	25.5b	50.6a	2277ab	25.4a	14.9ab	1129a
15	27.6b	35.6b	2453ab	16.0a	8.4b	870a
23*	47.5a	47.4a	3455a	19.1a	9.2ab	1098a
24*	29.0ab	39.7b	1756b	18.9a	7.7b	881a
26*	20.8b	38.9b	1911b	17.2a	6.3b	781a
27	23.8b	37.6b	2074b	17.6a	7.9b	921a
uninoculated	25.9b	43.9b	1741b	8.9b	7.2b	369b

* Strains selected for further study.

[+]Within a column, numbers followed by the same letter are not significantly different according to least significant difference test ($P \geq 0.05$).

Soil-based:

Three of the AAFC strains (#23, #24, #26) and one from the University of Minnesota collection (#12) were further tested in a soil system based on their performance in the Leonard jar experiment. Only strain # 23 stood out among the other strains as a potentially superior strain. Strain # 12 was included because of the high shoot N contents in plants inoculated with it, and strains 24 and 26 were included because of the enhancement in root growth achieved in plants inoculated with these two strains. The very slow growth of these AAFC strains, may affect the time needed for optimal N fixation to be achieved.

Consistent with results from the Leonard jar experiment, plants inoculated with strain # 23 had the highest productivity (Table 5) and overall N uptake (Table 6). Strain # 24 also performed well, especially in terms of root production and overall N uptake. Interestingly, neither of these strains induced more nodules than the other strains nor the uninoculated control plants. Instead the nodules that were produced appear to be more metabolically active in N fixation with these strains. Numerous small nodules can be as effective as a few large nodules; size is not an accurate indicator of N fixation capacity. Similarly, nodules of equal size can differ in activity, and is especially apparent in ineffective nodules that do not develop adequate enzymatic pathways for N fixation. For example, white ineffective nodules do not develop adequate amounts of leghemoglobin to maintain optimal oxygen levels for N fixation. While nodules form, they are not active in fixing N from the atmosphere (Vincent, 1970). A few nodules were observed on the root systems of uninoculated controls (Table 5) indicating that some rhizobia were present in the soil that are capable of nodulating sainfoin or possibly that some contaimination of the control plants occurred from the other strains. However, the very low biomass and N uptake numbers indicate that the nodules are probably completely

inactive. The induction of ineffective nodules is not uncommon and can occur at the farm level when the wrong type of inoculant is used to inoculate a legume crop; for example if a chickpea inoculant is used to inoculate lentil. The overall low numbers of nodules detected on the root systems of plants grown in the soil-based experiments (Table 5) might indicate a problem with microbial competition with these strains in the soil environment. Nodule numbers were much lower in the soil experiment compared to the Leonard jar experiment. However, biomass production and tissue N contents and N uptake indicate that the plants are acquiring sufficient N for plant growth. Soil experiments typically under report nodule numbers because during the harvesting of roots, nodules are sheered off and not accounted for. The relatively low numbers of nodules are probably at least partially due to unaccounted for nodules.

We can get an indication of N-fixation in plants inoculated with the different strains, by examining the total N acquisition compared to the controls. Strains #23 and 24 aquired the most N compared to the controls which can be assumed to be due to superior N fixation. However, it was beyond the scope of this study to estimate N-fixation directly. Instead this study served as a first step in identifying a strain or strains of rhizobia that effectively nodulate sainfoin and have the potential to induce a high level of N fixation.

Table 5: Productivity of sainfoin grown in soil with selected rhizobial strains. Plants were grown for 10 weeks. Numbers are averages per plant.

Strain	Root Weight (g)	Shoot Weight (g)	Nodule Weight (μg)	Nodule Number
12	2.5b	0.8b	6.0	5.1
23	6.1a	1.4a	14.1	6.1
24	4.7ab	1.5a	19.2	13.5
26	1.8b	0.7b	8.0	4.3
uninoculated	1.7b	0.7b	1.0	1.5

[+]Within a column, numbers followed by the same letter are not significantly different according to least significant difference test ($P \geq 0.05$).

Table 6: Nutrient (Nitrogen and Phosphorus) contents of Sainfoin grown in soil and inoculated with selected rhizobial strains. Plants were grown for 10 weeks. Numbers are averages per plant.

	N content (mg g^{-1} tissue)		N uptake (g $plant^{-1}$)	P content (mg g^{-1} tissue)		P uptake (g $plant^{-1}$)
Strain	**Root**	**Shoot**		**Root**	**Shoot**	
12	10.4a	17.7ab	14.7b	1.9	1.7	1.7
23	6.8ab	15.0ab	68.4a	1.5	1.6	10.3
24	8.0a	24.3a	45.4a	1.3	1.8	4.6
26	9.0a	8.2b	20.2b	1.6	1.5	4.3
uninoculated	5.4b	13.9ab	19.9b	1.4	1.6	2.7

[+]Within a column, numbers followed by the same letter are not significantly different according to least significant difference test ($P \geq 0.05$).

e) ***Conclusions and Recommendations***

Of the 36 original rhizobial strains screened for their ability to nodulate sainfoin, two (strains #23 and #24) were identified as potentially superior strains based on the overall N acquisition by plants inoculated with these strains. Plants inoculated with these strains contained between 2.3 X (strain #24) and 3.4 X (strain # 23) more N than the uninoculated control and the other strains tested. Both of these strains originated from the AAFC culture collection in St. Foy Quebec. These strains originate from arctic soils, and hence have an inherent cold tolerance, which may prove to be beneficial in a commercial inoculant for Canadian producers. This also provides additional challenges, as these cultures were very slow to establish and more difficult to maintain than cultures developed from the other strains.

Further testing is essential to determine directly the amount of N fixed by plants nodulated with these strains using ^{15}N isotope dilution methodology. Furthermore, the short- and long-term competitiveness of the strains in a variety of soil types is essential to determine in field situations. Ideally experiments using molecular markers and/or fatty acid methyl ester (FAME) profiles to follow the survival of the introduced species in the soil and occupancy in nodules, would provide unequivocal evidence of the strain competitiveness. Because sainfoin is a perennial crop it is essential that the N-fixation capacity of the plant be sustained through multiple growth seasons.

f) ***Acknowledgements:***

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g) ***Literature Cited***

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k) ***Appendices*** – none -

i) ***Administrative and Other Aspects***

Personnel: Morgan Sather – summer student - full time 4 months

Janet Foley – technician – half –time 6 months

Mark Cooke – technician – half-time 12 months